CHAPTER

09

CHROMATOGRAPHIC METHODS TO ISOLATE MARINE NATURAL PRODUCTS FROM SEAWEEDS

Kajal Chakraborty, Shubhajit Dhara, Angel Saijan, Nisha Alex, Silpa K P. & Anjana A.G.

Marine Biotechnology Fish Nutrition and Health Division, Central Marine Fisheries Research Institute, Post Box No. 1603, Ernakulam North P.O., Kochi-682 018

ABSTRACT

Chromatography depends on creating a dynamic balance in solute concentrations between two phases. This continuous equilibrium entails the consistent migration of solute molecules between the two phases. The ratio of concentrations in this equilibrium is known as the distribution coefficient, and the term "affinity" denotes the attraction to a specific phase. For example, a compound moving at a slower rate is characterized by a stronger affinity for the stationary phase in comparison to one moving more swiftly. The affinity of a compound for a given phase is contingent upon the attractive forces between the molecules of the compound and those of the phase.

Keywords: Chromatography, Separation, Stationary and mobile phases, HPLC, GC

CHROMATOGRAPHY: AN OVERVIEW

Chromatography is primarily a separation technique extensively employed in chemical analysis. Nonetheless, to a limited extent, it is also utilized for preparative purposes, particularly isolating relatively small quantities of materials with comparatively high intrinsic value. In a one-step process, it can effectively separate a mixture into its individual components while simultaneously offering a quantitative assessment of each constituent. Samples may be in the form of gases, liquids, or solids, varying in complexity from a basic blend of two enantiomers to a multi-component mixture containing chemically diverse species. The Russian botanist Tswett was the first scientist to acknowledge chromatography as an effective method of separation. He employed a basic liquid-solid chromatography technique to separate various plant pigments. The colored bands that he generated on the adsorbent bed led to the term chromatography being coined for this particular type of separation, often referred to as "color writing". Even though color plays a minimal role in modern chromatography, the name has endured. Despite its lack of relevance, it continues to be applied to all separation techniques that incorporate the fundamental elements of chromatographic separation, namely a mobile

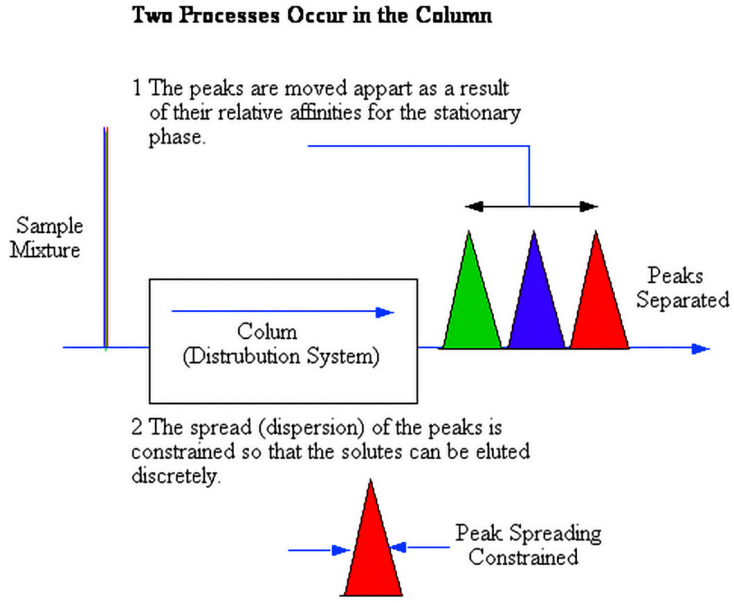
phase and a stationary phase. Presently, chromatography stands as an exceptionally versatile technique capable of separating gases and volatile substances through gas chromatography (GC), as well as in-volatile chemicals and materials with extremely high molecular weight, including biopolymers, through liquid chromatography (LC). Chromatography is a separation process accomplished by partitioning the components of a mixture between two phases: a stationary phase and a mobile phase. Components that are preferentially held in the stationary phase are retained in the system for a longer duration compared to those selectively distributed in the mobile phase. As a result, solutes are eluted from the system with local concentrations in the mobile phase based on their increasing distribution coefficients concerning the stationary phase. Consequently, a separation is achieved. Major types of forces involved in chromatographic process are listed in Table 1

Types of intermolecular force involved in chromatographic processes

Functional groups in molecules	**Type of intermolecular force**	**Strength of intermolecular force**
Hydrocarbon chains	Van der Waals interactions	Weak
C=C double bonds, Halogens, nitrogen	Dipolar and induced dipolar attractions	Weak
OH, NH groups	Hydrogen bonds	Moderately strong
Ionizable or charged groups	Ionic attractions	Strong

In practical terms, the distribution system, which refers to the portion of the chromatographic apparatus where solutes distribute between the phases, may manifest as a column, such as a tube filled with particulate matter where the stationary phase is bonded or coated. The mobile phase, which can be a gas or a liquid, flows under pressure through the column to elute the sample. Alternatively, the column may take the form of a long, small-diameter open tube with the stationary phase coated or bonded to the internal surface. Alternatively, the chromatographic system can be in the form of a plate, typically made of glass, with its surface loaded with particulate matter to which the stationary phase is coated or bonded. The mobile phase, typically a liquid, is directed to percolate up the plate, often driven by surface tension forces, to elute the sample. The sample is introduced into the mobile phase stream just before the leading edge of the column. The column is structured to facilitate two processes that contribute to the separation. Firstly, due to distinct forces between each molecular type and the stationary phase, each solute is retained to a varying degree. Consequently, those less

strongly held will elute first, while the more strongly held ones will elute last. The process is illustrated diagrammatically below.



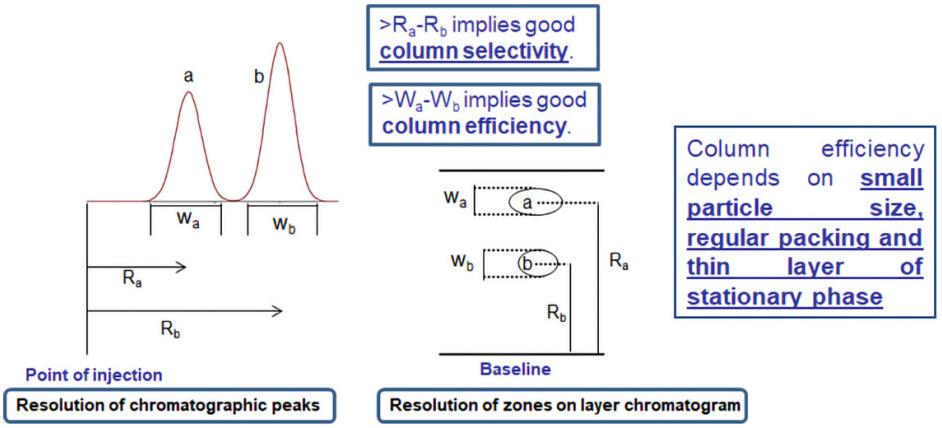
CHROMATOGRAPHIC RESOLUTION

In most chromatographic separations it is necessary to achieve good resolution. Resolution is the assessment of the separation of two peaks or zones and depends on two factors: the distance between their points of maximum concentration and the width of each zone. It is given by this formula:

$$R = \frac{R_a - R_b}{(w_a - w_b)/2}$$

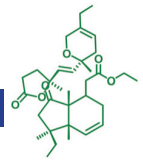
The distance between the two zones (Ra - Rb) reflects the selectivity of the column and relies on the relative distribution coefficients of its components; the greater the disparity, the wider the gap between the two zones. This distance can be adjusted by modifying one or both of the phases. Another aspect of resolution, column efficiency, is linked to the width of each peak (wa - wb). Achieving good column efficiency necessitates several factors: employing a small particle size for the stationary phase or its support, ensuring the stationary phase is tightly

packed within a narrow size range, applying a thin layer of stationary phase, maintaining an optimal flow rate, and controlling the temperature.



Summary of chromatographic process and techniques

Chromatographic technique	Process	General stationary phase	Mobile phase
Column, thin-layer (TLC), some HPLC	Adsorption	Silica gel (dry), alumina, polyamide (H bonding)	Non-polar or wet liquids
Paper, TLC, liquid-liquid, gas chromatography (GC)	Partition	Paper, cellulose, specialized phases for GC	Polar or moderate liquids, gas
High-performance liquid chromatography (HPLC)	Reversed phase-partition	Bonded silicas,	Polar or moderate liquids
Column, HPLC	Ion-exchange	Specialized resins	Aqueous
Column, HPLC	Size exclusion	Specialized polymers (sephadex)	Aqueous, moderate polar organic liquids
Column	Affinity	Specialized gels with attached ligands	Aqueous



CLASSIFICATION OF CHROMATOGRAPHY

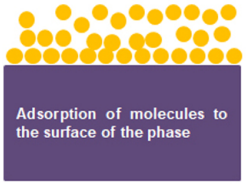
Since all chromatographic separations involve both a mobile and a stationary phase, the fundamental classification of chromatography is determined by the physical nature of the mobile phase. This distinction gives rise to two basic forms of chromatography: gas chromatography (GC) and liquid chromatography (LC). The stationary phase can also exist in two forms: solid and liquid, leading to two subgroups each for GC and LC namely, gas–solid chromatography (GSC) and gas–liquid chromatography (GLC), as well as liquid–solid chromatography (LSC) and liquid chromatography (LLC). The following table provides a summary of the various forms of chromatography. It's worth noting that most thin-layer chromatography techniques are categorized as liquid-solid systems, although the solute typically interacts with a liquid-like surface coating on the adsorbent or support, or in some instances, an actual liquid coating.

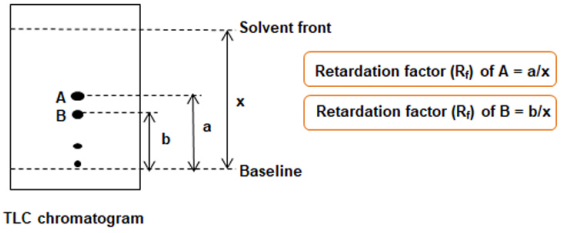
The classification of chromatography

Mobile phase	Stationary phase
Gas	Liquid
Gas Chromatography (GC)	Liquid Gas-liquid chromatography (GLC)
	Solid Gas Solid Chromatography (GSC)
Liquid	Liquid
Liquid chromatography (LC)	Liquid –liquid chromatography (LLC) Solid Liquid solid chromatography (LSC)

ADSORPTION CHROMATOGRAPHY

Adsorption is the process where a substance is distributed between the surface of a solid and a liquid in which it is soluble. It is postulated that a limited number of sites are present on the solid surface to which molecules adhere. Consequently, when the solution is overly concentrated, a constant equilibrium cannot be achieved as all the sites are occupied, leading to an excessively high concentration in the liquid phase. In conventional thin-layer chromatography and column chromatography, adsorption is the primary mechanism driving separation. The adsorbent is composed of fine particles forming a powder that is either spread as a layer or packed into a tube.





THIN LAYER CHROMATOGRAPHY

Thin layer chromatography (TLC) was introduced pioneered by Russian scientists N.A. Izmailov and M.S. Schreiber in 1938. It has become a crucial technique in analytical chemistry. The separation in TLC hinges on the relative affinity of compounds for both phases involved. As the compounds in the mobile phase traverse the surface of the stationary phase, those with a greater affinity for the stationary phase move at a slower pace, while others progress more swiftly. This differential movement leads to the appearance of distinct spots on the TLC plates, each corresponding to an individual component of the mixture. TLC serves as a valuable tool for qualitative analysis, offering a visual representation of the separation process and aiding in the identification of various components in a given sample.

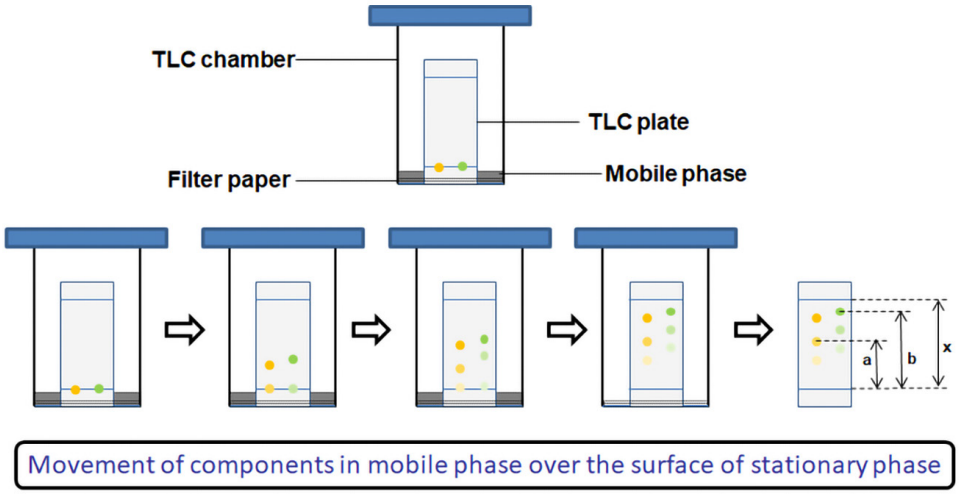
MAJOR COMPONENTS OF TLC ARE:

TLC plates: Comprising a stationary phase, these plates are stable and chemically inert, featuring a thin layer of stationary phase with a thick consistency and fine particles applied uniformly across the entire surface.

TLC chamber: It is employed in the development of TLC plates to establish a consistent environment conducive to the optimal development of spots. Moreover, it prevents solvent evaporation and ensures a process free from dust contamination.

Mobile phase: The mobile phase comprises a solvent or a blend of solvents. It is imperative that the mobile phase used is devoid of particles and of utmost purity to enable precise development of TLC spots. The suggested solvents should be chemically inert regarding both the sample and the stationary phase.

Filter paper: Before being placed inside the chamber, this is moistened with the mobile phase to aid in generating a uniform rise of the mobile phase along the length of the stationary phase.



Movement of components in mobile phase over the surface of stationary phase

$$\text{Retardation factor } (R_f) = \frac{\text{Distance travelled by the component}}{\text{Distance travelled by the solvent}}$$

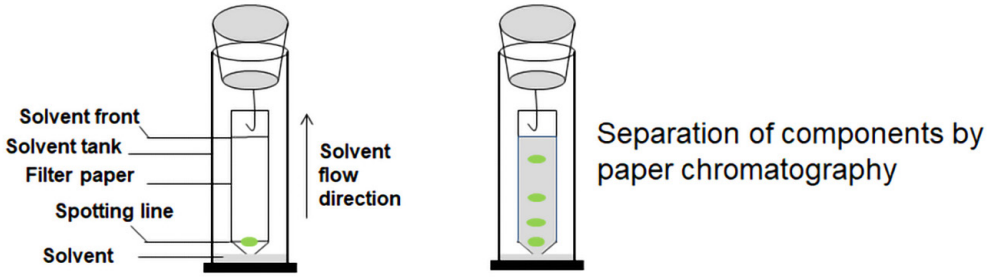
APPLICATION OF THIN LAYER CHROMATOGRAPHY

Thin layer chromatography (TLC) finds wide application across various fields due to its versatility and effectiveness. One common use is monitoring the progress of chemical reactions in laboratories, allowing researchers to observe the separation of compounds over time. Additionally, TLC is utilized to determine the composition of mixtures by separating and identifying individual compounds present. It serves as a valuable tool in assessing the purity of substances, enabling researchers to detect impurities or contaminants within a sample. Moreover, TLC facilitates the identification of specialized components within a mixture through the application of different chromogenic reagents, which react with specific compounds, producing visible spots that aid in their identification and characterization. Overall, TLC is a versatile technique with diverse applications in research, quality control, and analytical chemistry.

PAPER CHROMATOGRAPHY

Paper chromatography was conceived by Archer John Porter Martin and Richard Laurence Millington Synge. This technique is very useful in the field of analytical chemistry by addressing the challenge of separating closely related amino acids. This technique utilizes specialized

papers, typically highly purified, fine cellulose-based papers. In paper chromatography, substances distribute between a stationary phase and a mobile phase. The cellulose layers in filter paper, containing moisture, act as the stationary phase, while organic solvents are commonly employed as the mobile phase. As the components of the sample travel through the paper, they separate based on their affinity for adsorption onto the stationary phase versus their solubility in the mobile phase. Paper chromatography is widely used for separating mixtures in various industries such as pharmaceuticals, food, and cosmetics, as well as in biochemical laboratories, making it a versatile and valuable technique in analytical chemistry.



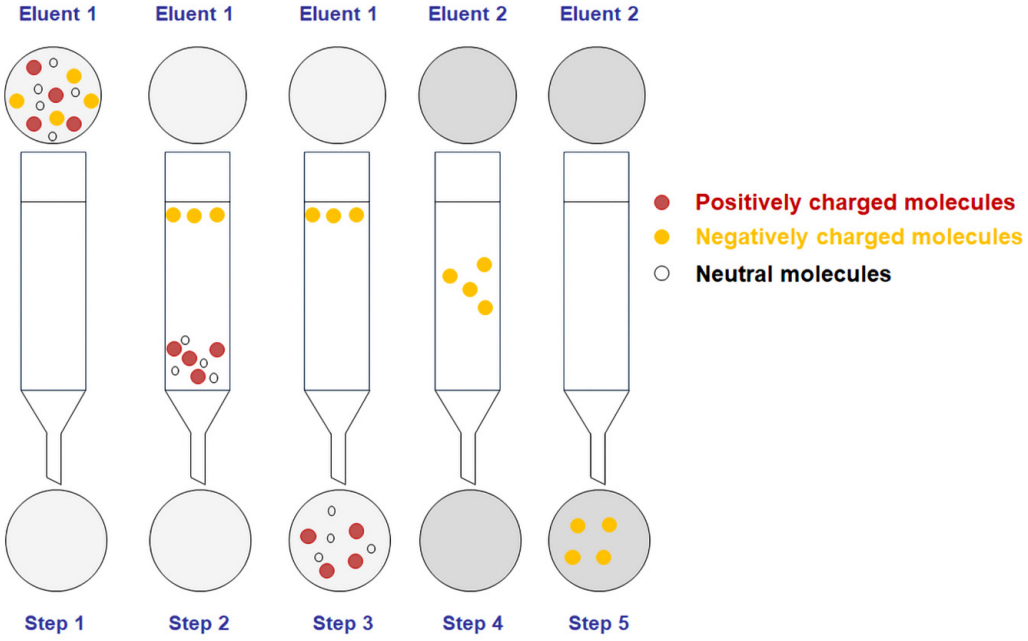
ION-EXCHANGE CHROMATOGRAPHY

Ion-exchange chromatography operates as a type of adsorption process where the stationary phase comprises a porous matrix, often referred to as a 'resin,' through which the mobile phase flows. This matrix consists of a cross-linked polymer to which ligands, carrying either a positive or negative electrical charge, are attached. Compounds with an opposite electrical charge, dissolved in the mobile phase (typically an aqueous buffer solution), are attracted to the charged groups on the stationary phase, establishing equilibrium in the concentrations of such molecules between the two phases. The affinity of a substance for the stationary phase is influenced by several factors, including the ionic strength and pH of the mobile phase, temperature, and the nature of the group carrying the opposite charge. Below is a table listing commonly used exchange resins and their major chemical groups.

Table 4. Types of exchange resins and major chemical group

Type of exchange resins	Chemical group	Conditions
Weak cationic (anion exchange)	Polyamine on polystyrene, amine on polystyrene, diethylaminoethyl dextran, diethylaminoethyl cellulose	pH 0-7, size of molecules < 1 × 106 daltons

Strong cationic (anion exchange)	Quaternary ammonium on polystyrene, Trimethylbenzyl-ammonium on polystyrene	pH 0-14
Weak anionic (cation exchange)	Carboxylic acid on polystyrene, carboxymethyl on dextran, carboxymethyl cellulose, polyacrylic acid	pH 4-14, size of molecules < 3 × 104 daltons
Strong anionic (cation exchange)	Sulphonic acid on polystyrene	pH 0-14



Steps involved in ion-exchange chromatography (in case of cation exchange resin)

Step 1: Addition of test compound in eluting solvent.

Step 2: Retention of negatively charged molecules by anionic stationary phase and movement of positively and neutral charged molecules.

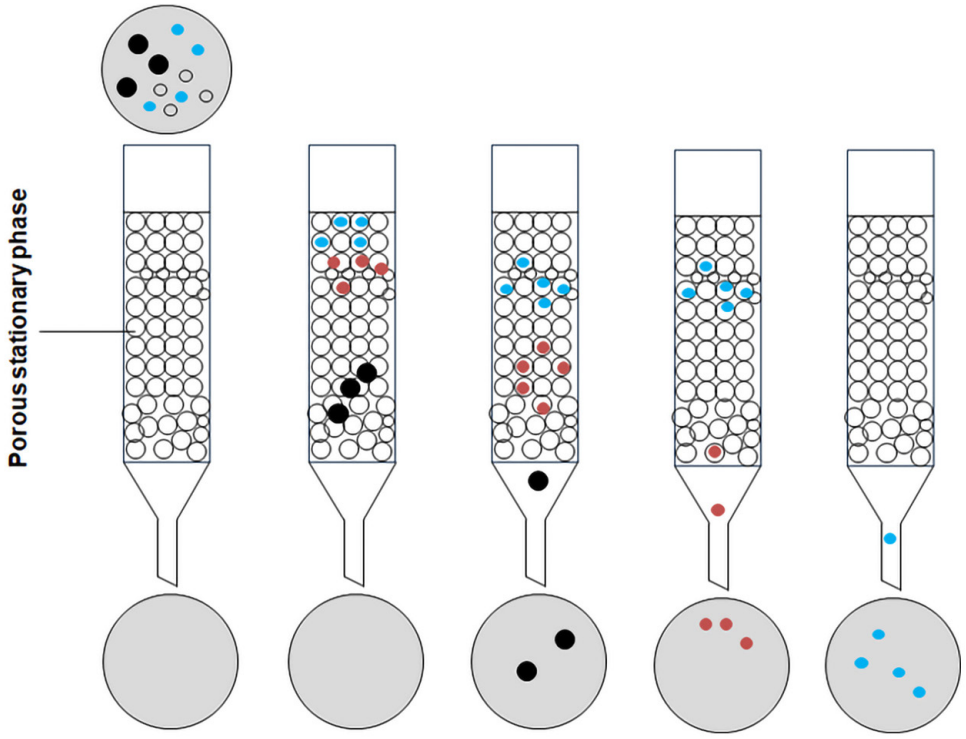
Step 3: Elution of positively and neutral charged molecules by solvent 1.

Step 4: Addition of solvent 2 for the elution of negatively charged molecules (with higher affinity for the stationary phase than negatively charged molecules).

Step 5: Elution of negatively charged molecules.

SIZE EXCLUSION CHROMATOGRAPHY

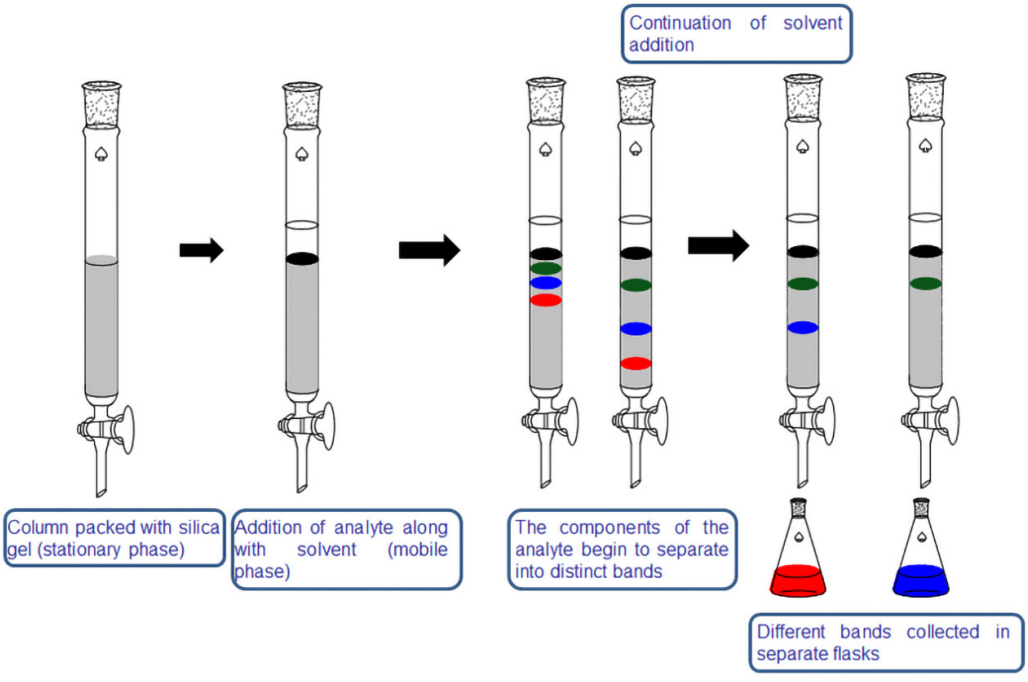
Size exclusion chromatography is also known as gel filtration or gel permeation chromatography. In this process, separation occurs based on the different sizes, and to some extent, the different shapes of the molecules dissolved in the mobile phase and applicable to molecules of > 300 daltons. Due to their size, large molecules cannot penetrate the pores, preventing them from entering the stationary phase and allowing them to traverse the chromatographic system rapidly. Smaller molecules can enter the pores and penetrate the mobile phase. Since the pores cover a range of sizes below the maximum, the smallest molecules enter more pores than those of larger size. Therefore the equilibrium is more in favour of the stationary phase for smaller and more spherical molecules, with the consequence that these types of compound have the longest retention times. In this process, crosslinked dextran (with epichlorohydrin/ hydroxypropyl) is generally used as stationary phase.



Pictorial representation of size-exclusion chromatography process

CONVENTIONAL COLUMN CHROMATOGRAPHY

Column Chromatography serves as a preparative method employed for the purification of compounds, relying on their polarity or hydrophobicity. In this technique, a mixture of molecules is separated by exploiting the varying partitioning behaviors between a mobile phase and a stationary phase. Columns can be either dry packed or wet packed. In dry packing the solid stationary phase is introduced into the column and allowed to settle. This can be aided by gently tapping the bottom end of the column on a hard surface and also tapping the walls with flexible rod. It is difficult to achieve a narrow band of applied sample with dry packing, and it is difficult to achieve uniform flow of the mobile phase because it is difficult to attain even packing of the stationary phase. In wet packing, stationary phase is suspended in the chosen mobile phase or one with lower polarity, ensuring a well-stirred and pourable suspension. Following this, the column is securely positioned upright, with the tap closed to prevent air gap formation. The suspension is poured into the column with care, and gentle tapping encourages settling while removing air bubbles. Finally, the tap is slowly opened to let the mobile phase run out until the height of the supernatant liquid is below 2 cm, avoiding a level below the stationary phase to maintain an even flow of the mobile phase. This meticulous procedure ensures a well-prepared chromatography column for effective substance separation.



GAS LIQUID CHROMATOGRAPHY

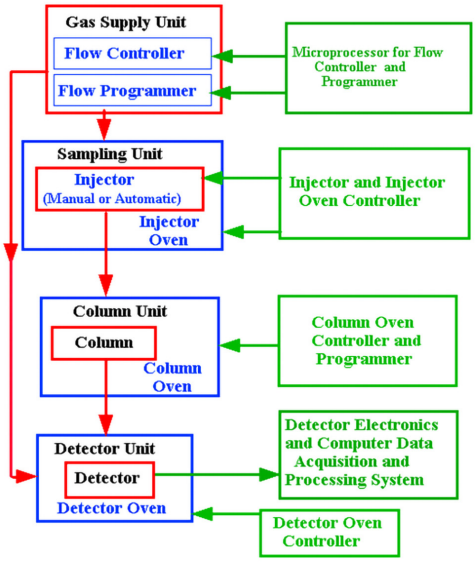
Gas-liquid chromatography (GLC) was invented by James and Martin as a chromatographic separation technique wherein the mobile phase is a gas, typically helium or nitrogen, and the stationary phase is a liquid. In the original columns employed by James and Martin, the liquid stationary phase was adsorbed on the surface of an inert support, such as Celite (a diatomaceous earth) or calcined Celite (a form of brick dust). Before use, the support was commonly deactivated through acid treatment and subsequent reaction with hexamethyldisilazane. This technique found extensive application in separating a diverse range of volatile substances, including fatty acids.

The contemporary gas chromatograph is a sophisticated instrument primarily controlled by a computer. Mechanically injected samples undergo automated analysis, with the calculated results and relevant operating conditions printed out in a standard format. Despite its current complexity, the instrument has undergone evolution over many years. The majority of additional devices and techniques were proposed or described during the first three international symposia on gas chromatography held in 1956, 1958, and 1960.

THE DIFFERENT COMPONENTS OF GLC

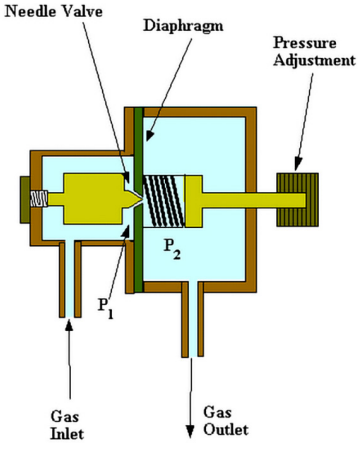
Gas supplies

Originally, gases (carrier gas such as N2 or He, and fuel gases like air and H2) used in gas-liquid chromatography were sourced from gas cylinders equipped with reducing valves. These valves were set to deliver the gas to the instrument at the recommended pressure specified by the manufacturers. The reducing valves on the gas cylinders exemplify simple pressure controllers, and the flow controllers used for detector and column flow regulation often employ devices based on similar principles. The pressure controller essentially consists of two chambers separated by a diaphragm, with a needle valve at the center that is operated by the diaphragm.



The layout of the modern gas chromatograph illustrated in the form of a block diagram

The diaphragm is pressed down by an adjustable spring, allowing the pressure in the second chamber and, consequently, the outlet flow to be configured to a chosen value. As gas enters the lower chamber, the pressure on the lower section of the diaphragm counteracts the spring setting, causing the valve to open. The gas then flows into the upper chamber, building pressure until it reaches the preset value. At this point, the diaphragm moves downward, closing the valve. If the pressure drops in the upper chamber, the diaphragm moves upward again due to the pressure in the lower chamber, reopening the valve and restoring the pressure in the upper chamber to its set value. A diagram of a pressure controller is depicted below:

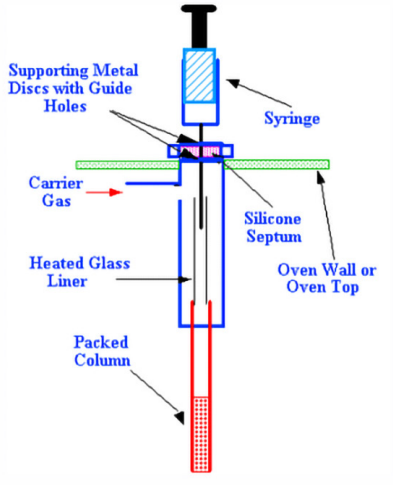


Pressure controller

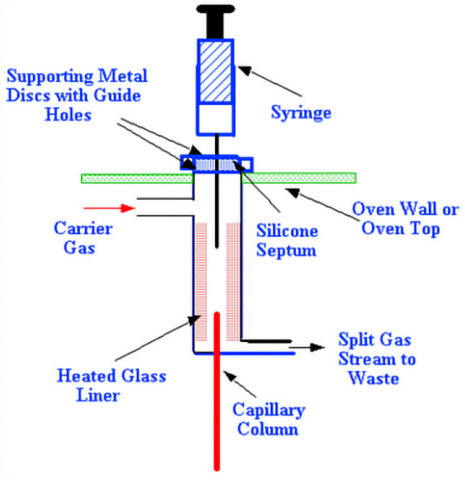
INJECTORS

The sample is introduced using a hypodermic syringe through a silicone rubber septum directly into the column packing or a flash heater. An illustration of a septum injection system employed for packed columns is presented in the following figure. The silicone septum undergoes compression between metal surfaces, allowing a hypodermic needle to pierce it. Upon withdrawal, the hole closes due to septum compression, preventing gas leaks. To minimize the likelihood of thermal decomposition, the glass liner shields the sample from direct contact with the heated metal wall. Additionally, the glass liner can be equipped with a separate heater, enabling control of the volatilization temperature. Employing a syringe with an extended needle allows the tip to penetrate beyond the liner, facilitating the direct discharge of contents into the column packing. This method, known as 'on-column injection,' is often preferred as it diminishes peak dispersion during injection, resulting in higher column efficiencies.

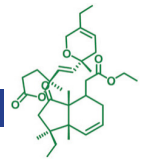
The fundamental distinction between the two injection systems lies in the capillary column extending into the glass liner, with a portion of the carrier gas sweeping past the column inlet to waste. When the sample traverses the column opening, a minor fraction is diverted and flows directly into the capillary column, hence this apparatus is termed a split injector. The split ratio can be adjusted by regulating the proportion of carrier gas directed to waste, achieved through an adjustable flow resistance in the waste flow line. This mechanism is employed specifically for small-diameter capillary columns where the quantity of the sample is crucial. Consequently, quantitative analyses conducted using high-efficiency small-diameter capillary columns may exhibit limited accuracy and precision, contingent on the nature of the sample.



Packed Column Injector



Split Injection System



GLC COLUMNS

There are two commonly used column types in gas chromatography (GC): the conventional packed column and the open tubular column. The former typically has an inner diameter (I.D.) of 2 to 4 mm and a length of 1 to 4 m. Packed with a suitable adsorbent, these columns are primarily employed for gas analysis. Due to the simpler injection procedure and more precise sampling method, the packed column tends to offer greater quantitative accuracy and precision. Despite challenges associated with sample injection, the open tubular column is regarded as the 'state of the art' column and is the most widely used column system. Open tubular or capillary columns can range in length from about 10 to 100 m and have internal diameters between 100 and 500 μm. The stationary phase is coated on the internal wall of the column as a film, typically 0.2 to 1 μm thick.

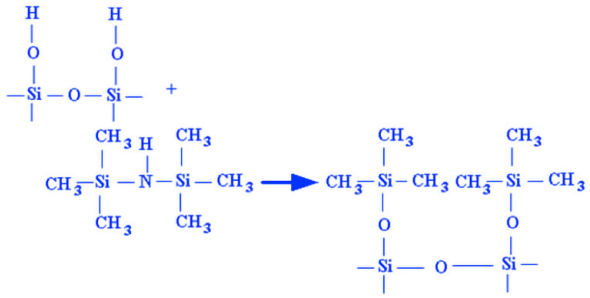
THE PACKED GC COLUMN

Packed columns are typically made from stainless steel or Pyrex glass. Pyrex glass is preferred when separating thermally labile materials, such as essential oils and flavor components. Longer columns can take a U-shaped form, but columns exceeding a meter in length are usually coiled. Glass columns are occasionally treated with a suitable silanizing reagent to eliminate surface hydroxyl groups, which can be catalytically active or result in asymmetric peaks.

SUPPORTS FOR GLC

Various materials have been employed as supports for packed gas chromatography (GC) columns, including Celite (a proprietary form of diatomaceous earth), fire-brick (calcined Celite), fire-brick coated with metallic silver or gold, glass beads, Teflon chips, and polymer beads. However, in contemporary packed gas-liquid chromatography (GLC) columns, the predominant choice for support materials is based on either Celtic or polystyrene beads. There are two processes used to modify Celite. In one approach, Celite is crushed, blended, and pressed into the form of a brick, then calcined at a temperature of about 900˚C. Under these conditions, some of the silica undergoes transformation into cristobalite, and traces of iron and other heavy metals interact with the silica, resulting in the material taking on a pink color. This material is marketed under the trade name Chromosorb P. The second process involves blending Celite with sodium carbonate and subjecting the mixture to flux at 900˚C. This disrupts the structure of Celite, and the resulting fragments adhere to one another through glass formed from silica and sodium carbonate. As the original Celite structure is disrupted, the material displays a wide range of pore sizes, distinct from the material calcined without sodium carbonate. This product is sold under the name Chromosorb W, along with two similar

materials known as Chromosorb G and Chromosorb S. The remaining undesirable adsorptive properties of the support arise from silanol groups on the surface, which can be eliminated through silanization. The support undergoes treatment with hexamethyldisilazane, replacing the hydrogen of the silanol group with a trimethylsilyl radical. The reaction proceeds as follows,



Through this process, the strongly polar silanol groups undergo methylation, adopting dispersive characteristics that eliminate peak tailing. While the primary contributors to adsorption by the support are the silanol groups, residual adsorption occurs due to trace amounts of heavy metals, such as iron. This can be substantially mitigated through acid washing before silanization.

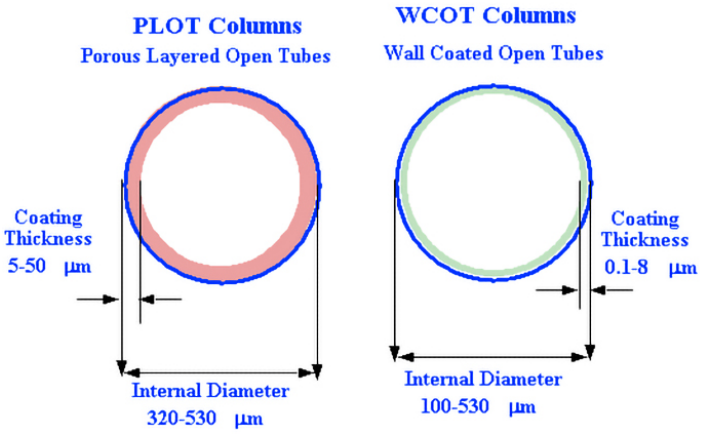
THE CAPILLARY OR OPEN TUBULAR COLUMN

Capillary columns are constructed from stainless steel, offering the high efficiencies associated with open tubular columns. They have been utilized for the analysis of various substances, including petroleum, fatty acids, and fuel oils. However, metal columns come with certain drawbacks. While they can be easily coated with dispersive stationary phases like squalane or Apiezon grease, they are less amenable to coating with more polar stationary phases such as CARBOWAX®. Moreover, the elevated temperatures of metal surfaces can lead to the decomposition or molecular rearrangement of thermally labile materials, such as the terpenes found in essential oils. Metal can also directly react with certain substances through chelation, adsorbing polar materials and resulting in asymmetric and tailing peaks. Despite these challenges, metal columns are durable, easy to handle, and simple to remove and replace in the chromatograph. Consequently, their use has persisted in various application areas, even with the advent of fused silica columns.

OPEN TUBULAR COLUMN TYPES

Open tubular columns are generally categorized into two classes: wall-coated open tubular columns (WCOT Columns), which have already been described and are by far the most popular, and porous layer open tubes (PLOT Columns). The two column types are illustrated

diagrammatically in the following figure. PLOT columns have an external diameter ranging from 320 to 530 µm, with a porous layer that can be 5 to 50 µm thick.

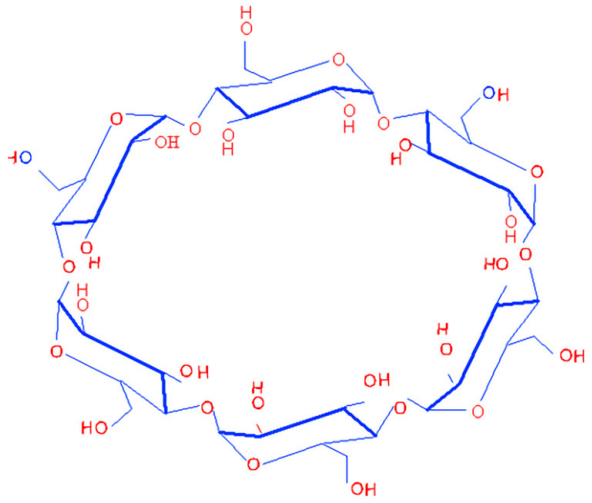


Open Tubular Column Types

CHIRAL STATIONARY PHASES

Modern organic chemistry and pharmaceutical research are increasingly focused on methods of asymmetric synthesis. This interest stems from the observed variations in physiological activity between the geometric isomers of pharmaceutically active compounds. A tragic example is the drug Thalidomide, which was initially distributed as a racemic mixture of N-phthalylglutamic acid imide. The crucial physiological activity resides in the R-(+)-isomer, and it was discovered too late that the S-enantiomer was likely teratogenic, causing severe fetal malformations. The separation and identification of isomers can be of utmost importance, and chromatography proves highly effective in resolving such mixtures. While the use of gas chromatography (GC) for separating asymmetric isomers is not as widespread as liquid chromatography (LC), there are nonetheless highly efficient optically active stationary phases suitable for GC in enantiomer separation. Some of the more useful GC stationary phases are based on cyclodextrins, as previously described. These columns are typically 30-60 meters long with a 0.25 mm inner diameter and operate within a temperature range of 30˚C to 250˚C. To utilize cyclodextrins as stationary phases for GC, permethylated cyclodextrins are often embedded in a siloxane matrix (e.g., 35% phenyl-65% methyl polysiloxane) deposited on the walls of fused quartz capillary tubes.

.



The Structure of Cyclodextrin

Modifying the fundamental cyclodextrin structure through derivatization allows for the introduction of groups that selectively interact with only one enantiomer, while the others are either partially or entirely entropically hindered from interaction. This augmentation amplifies the differential interaction between the enantiomers and the stationary phase, consequently enhancing the separation ratio and, consequently, the resolution.

COLUMN OVEN AND ACCESSORIES

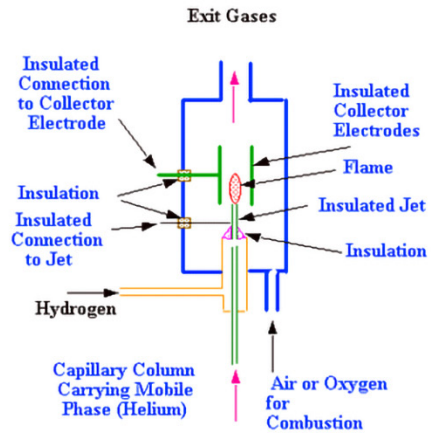
The column oven should be capable of operating within a broad temperature range, for example, from 5˚C to 400˚C. However, in practical applications, the maximum oven temperature required is typically below 250˚C, especially when using synthetic stationary phases, as many of them exhibit instability and may decompose or volatilize at higher temperatures. Similarly, initial temperatures below 50˚C are seldom necessary. The oven typically incorporates air circulation, facilitated by a robust fan, to ensure a uniform temperature across the entire oven. The temperature programmer, encompassing both hardware and software components, generally provides a range of linear gradients from 0.5˚C/min to about 20˚C/min. While some programmers include nonlinear programs such as logarithmic and exponential, most gas chromatography (GC) analyses can be effectively accomplished using linear programs only. The program rate can be adjusted at any point during chromatographic development, and intermittent isothermal periods can be inserted as needed in the program. The temperature programming limits are usually the same as those of the oven (viz. 5˚C to 400˚C).

GC DETECTORS

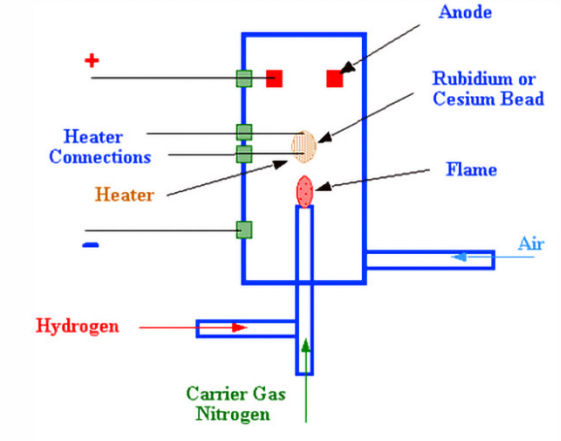
A multitude of gas chromatography (GC) detectors have been developed and are commercially accessible. Detectors that exhibit the highest sensitivity typically specialize in sensing specific types of samples, such as halogenated substances detected by the electron capture detector. Conversely, detectors with a broad response are more commonly used, and the flame ionization detector (FID) is widely employed for monitoring the majority of GC separations. Among the commonly used specific detectors are the nitrogen phosphorus detector (NPD) and the electron capture detector (ECD).

FLAME IONIZATION DETECTOR

The flame ionization detector (FID) employs hydrogen as the combustion gas, which is mixed with the column eluent (helium, nitrogen, or another appropriate gas) and ignited at a small jet positioned inside a cylindrical electrode. A potential of a few hundred volts is applied between the jet and the electrode. When a carbon-containing solute is burned in the jet, the electron/ion pairs formed are collected at the jet and cylindrical electrode. The resulting current is amplified and directed to a recorder or the A/D converter of a computer data acquisition system. Throughout the oxidation process, oxidized or partially oxidized fragments of the solute are generated in the flame, believed to produce electrons through thermionic emission. The background current (ions and electrons from the hydrogen flame alone) is very small (1-2 x 10-12 amperes) and consequently, the noise level is also commensurably small (about 10-14 amperes).



Flame Ionization Detector



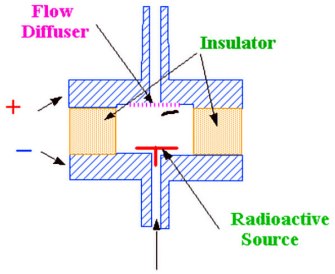
Nitrogen Phosphorus Detector

NITROGEN PHOSPHORUS DETECTOR

The nitrogen phosphorus detector (NPD) is a highly sensitive yet specific detector that directly evolved from the flame ionization detector (FID). It provides a robust response to organic compounds containing nitrogen and/or phosphorus. Despite appearing to function similarly to the FID, the NPD operates on an entirely different principle. The NPD sensor consists of a rubidium or cesium bead enclosed within a small heater coil. A potential is applied between the bead and the anode. The heated alkali bead emits electrons through thermionic emission, collected at the anode, thereby generating an ion current. When a solute containing nitrogen or phosphorus is eluted, the partially combusted nitrogen and phosphorus materials are adsorbed on the bead's surface. This adsorbed material reduces the work function of the surface, consequently increasing the emission of electrons and raising the anode current. The sensitivity of the NPD is about 10-12 g/ml for phosphorus and 10-11 g/ml for nitrogen).

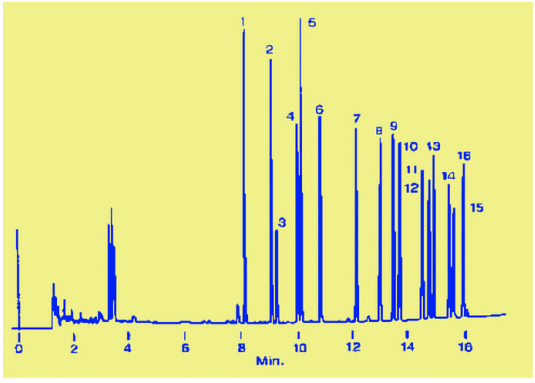
ELECTRON CAPTURE DETECTOR

The electron capture detector incorporates a low-energy β-ray source used to generate electrons for capture by suitable atoms. While tritium adsorbed into a silver foil has been utilized as the β particle source, it is relatively unstable at high temperatures, making the Ni63 source preferable. The detector can operate in two modes: either with a constant potential applied across the cell (DC mode) or with a pulsed potential across the cell (pulsed mode). In the DC mode, hydrogen or nitrogen can serve as the carrier gas, and a small potential (usually just a few volts) is applied across the cell, sufficient to collect all available electrons and provide a small standing current. When an electron-capturing molecule (such as a molecule containing a halogen atom with only seven electrons in its outer shell) enters the cell, the electrons are captured by the molecule, rendering it charged. The mobility of the captured electrons is significantly lower than that of free electrons, causing a dramatic reduction in the electrode current. During the inactive period of the waveform, electrons with thermal energy alone readily attach themselves to any electron-capturing molecules present in the cell, resulting in the production of negatively charged ions. These negative ions promptly recombine with the positive ions (simultaneously produced with the electrons by the β particles), rendering them unavailable for collection. Consequently, the standing current measured during the potential pulse is diminished.



Electron Capture Detector

The basic electron capture detector comprises a small chamber, typically one or two milliliters in volume, housing two metal electrodes. These electrodes may take the form of concentric cylinders or metal discs separated by an insulator. Within the cell, there is a radioactive source electrically connected to the entrance conduit and the negative side of the power supply. A gauze "diffuser" is linked to the cell exit and connected to the positive side of the power supply. The sensor's output is processed by suitable electronics and transmitted to either a potentiometric recorder or a computer data acquisition system. Renowned for its high sensitivity, the electron capture detector is likely the most sensitive gas chromatography (GC) detector available, approximately 10-13 g/ml, and is widely employed in the analysis of halogenated compounds.



Analysis of chlorinated insecticides

1	2	3	4 Heptachlor
5	6 Aldrin	7 Heptachlor Epox.	8 Endosulphan
9 p,p'-DDE	10 Dieldrin	11 Endrin	12 p,p'-DDD
13 Endosulphan 11	14 p,p'-DDt	15 Endin Aldehyde	16Endosulp. Sulf.

DATA ACQUISITION AND PROCESSING

Initially, analytical results were computed based on measurements taken directly from the chromatogram provided by the chart recorder. The output from the detector, which is seldom the direct output from the detector sensor, typically comes in millivolts and is suitable for direct connection to a potentiometric recorder. This output usually goes directly to a scaling amplifier, which adjusts the signal to a range suitable for the analog-to-digital (A/D)

converter. Alternatively, the output can be directed to a potentiometric recorder to generate the chromatogram in real time. The computer system also has the capability to produce a real-time chromatogram, but for this to occur, the data must be processed, and the chromatogram must be presented on the printer.

QUANTITATIVE ANALYSIS

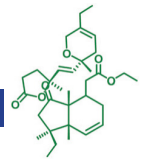
There are three important stages in a GC analysis,

1. The preparation of the sample.
2. The development of the separation and the production of the chromatogram
3. The processing of the data and the presentation of the results.

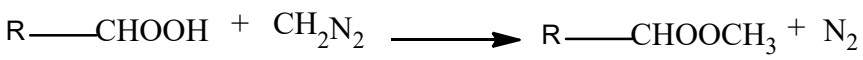
Each stage is of equal importance, and if not executed correctly, the results will lack precision and accuracy. Sample preparation can range from a straightforward process, such as diluting a known weight of the sample with a mobile phase, to a more intricate one involving extraction, derivatization, and subsequent dilution. Liquid extraction, especially on the micro scale often required in sample preparation, can be a cumbersome procedure. An alternative approach is solid-phase extraction, which is relatively straightforward and entails using a short tube packed with a suitable adsorbent like silica, reversed-phase silica, or, for certain applications, macro-porous polymer beads. The adsorbent must effectively remove the substances of interest from the liquid medium.

DERIVATIZATION

GC samples are usually derivatized to render highly polar materials sufficiently volatile so that they can be eluted at reasonable temperatures without thermal decomposition or molecular re-arrangement. Examples of such materials that need to be derivatized are the organic acids, amides, poly hydroxy compounds, amino acids etc. In order to render such materials more volatile, they are either esterified, silanated or acetylated using one of a number of different methods of derivatization. Acids can be esterified by treating them with an appropriate alcohol using an inorganic acid to catalyze the reaction. Hydrochloric acid was popular for this purpose because its strength was adequate and any excess could be easily removed. Other catalysts that have been found effective are trifluoroacetic acid, dichloroacetic acid, benzene sulphonic acid, p-toluene sulphonic acids and suphuryl and thionyl chlorides. A volatile acid is recommended such as hydrochloric acid or thionyl chloride. However, the derivative must be must be sufficiently involatile not to allow loss when removing the excess alcohol and where appropriate the catalyst itself. The Lewis acid boron trifluoride or the equivalent reagent



boron trichloride is also very useful for forming ester derivatives. Boron trifluoride is supplied as a 14% solution in methanol. Boron trifluoride catalyzed reactions are very fast and can be complete in a few minutes. The esters can be extracted with n-hexane with vigorous shaking. Another popular esterifying reagent is diazomethane. Diazomethane is a yellow gas but is used in the form of an ethereal solution. It reacts with an organic acid in the following manner,



Once the reaction is finished, the yellow color persists, making the reagent serve as its own indicator.

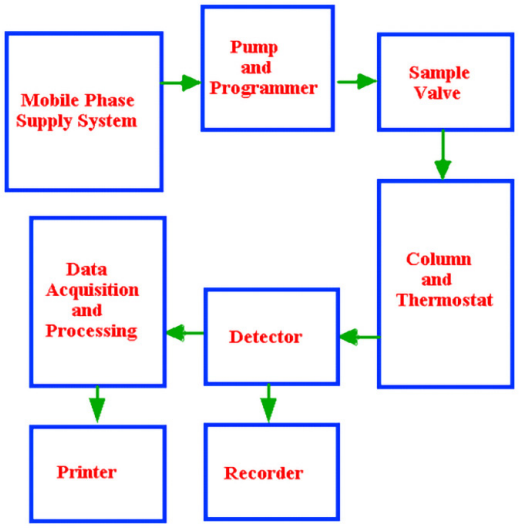
HIGH PRESSURE LIQUID CHROMATOGRAPHY

Liquid chromatography (LC) was the first chromatography type to be discovered. In the late 1890s, the Russian botanist Tswett initially used liquid-solid chromatography (LSC) to separate and isolate various plant pigments. The colored bands he generated on the adsorbent bed led to the term chromatography (color writing) for this separation method. In the late 1930s and early 1940s, Martin and Synge introduced a form of liquid-liquid chromatography by supporting the stationary phase (water in this case) on silica gel in the form of a packed bed, using it to separate some acetyl amino acids. Martin and Synge suggested employing small particles and high pressures in LC to enhance separation, which turned out to be critical factors that initiated the development of high-performance liquid chromatography (HPLC). The statement made by Martin in 1941 encapsulates all the necessary conditions for achieving both the high efficiencies and high resolution seen in modern LC columns. Despite these recommendations, it has taken nearly fifty years to bring his concepts to fruition. The primary obstacle to LC development was the absence of a highly sensitive detector. It wasn't until the refractive index detector was developed by Tiselius and Claesson in 1942 that the technique could be effectively advanced. However, the contemporary chromatograph is now a sophisticated instrument operating at pressures up to 10,000 PSI, providing flow rates ranging from a few microliters per minute to 10-20 ml/minute, depending on the type of LC being conducted. Modern detectors can detect solutes at concentration levels of 1x10-9 g/ml, and an analysis can be completed in a few minutes with just a few micrograms of sample.

MODERN HIGH PRESSURE LIQUID CHROMATOGRAPHY (HPLC)

High-Performance Liquid Chromatography (HPLC) is a form of liquid chromatography that has been fine-tuned to deliver swift, high-resolution separations. The fundamental liquid chromatograph comprises five primary units, outlined as follows. A block diagram of the basic liquid chromatograph is presented in the figure below.

1. Mobile phase supply system and gradient mixers.
2. HPLC high pressure pumps and sample valves.
3. HPLC columns with inert packing materials.
4. High sensitivity low dispersion HPLC detectors.
5. High speed data acquisition systems.



The Basic Liquid Chromatograph

MOBILE PHASE SUPPLY SYSTEM AND HPLC GRADIENT MIXERS

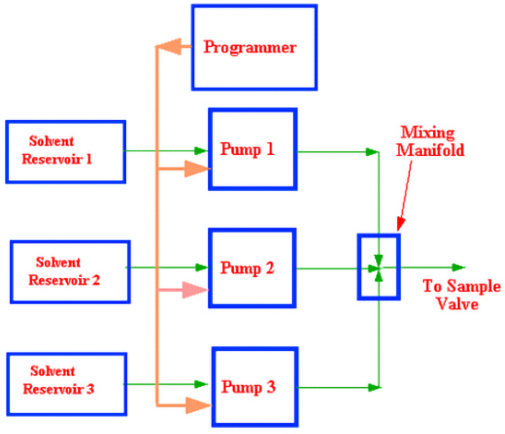
HPLC gradient mixers offer precise control of solvent composition, ensuring a reproducible gradient profile. The mobile phase supply system comprises several reservoirs (200-1,000 ml), typically made of glass or stainless steel, each with an exit port open to the air. A gas diffuser, allowing the bubbling of helium, is usually attached to each reservoir. Many solvents, especially aqueous mixtures, contain dissolved nitrogen and oxygen from the air. These gases can create bubbles in the chromatographic system, leading to significant detector noise and column efficiency loss. Helium, being largely insoluble in most solvents, purges oxygen and nitrogen without causing bubbles in the system. Using a vacuum on the reservoir is not a permanent solution to dissolved air, as air can re-dissolve when the vacuum is released for the solvent to reach the pump. The solvent undergoes filtration through a stainless steel or sintered glass filter to eliminate solid contaminants. Depending on the solvent programmer type, the supply from each reservoir may flow either to a pump or to a valve blending device.

Solvent reservoirs are typically not thermostatted, but if necessary, the solvent can be brought to the column temperature using an appropriate heat exchanger.

THE GRADIENT PROGRAMMER

HIGH PRESSURE GRADIENT PROGRAMMER

There are two fundamental types of solvent programmers. In the first type, solvent mixing takes place at high pressure, while in the second type, solvents are pre-mixed at low pressure before being directed to the pump. In theory, a mobile phase program can involve any number of solvents; however, the majority of LC analyses typically require only two solvents. Nonetheless, the system can accommodate up to four solvents. The layout of a high-pressure gradient system is depicted in the figure below, which, as an example, illustrates the provision for three solvents to be mixed through appropriate programming.



High Pressure Gradient Programmer

Solvent flows from each reservoir directly to a pump, then to a mixing manifold, and finally to the sample valve and column. The pumps govern the program, typically operated by stepping motors. The volume delivery of each solvent is regulated by the pump's speed, precisely determined by the frequency of its power supply. This controlling frequency can be generated either by external oscillators or, if the chromatograph is computer-controlled, directly from the computer.

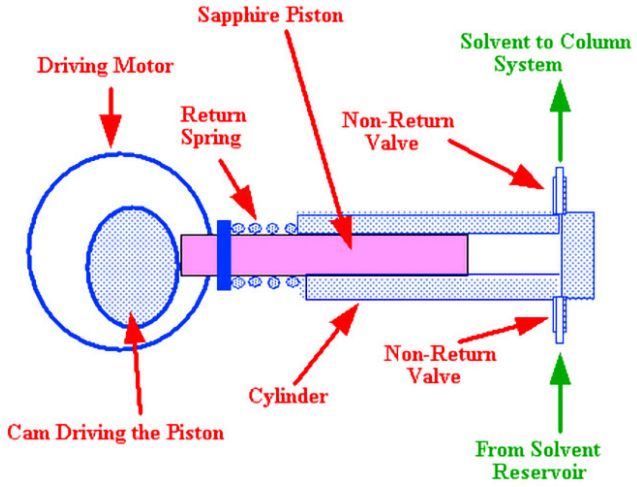
HPLC PUMPS

Due to the use of small particles in modern HPLC, LC pumps must operate reliably and precisely at pressures of 10,000 PSI or at least 6,000 PSI. To operate at these pressures

and maintain compatibility with a wide variety of solvents used in HPLC, pumps typically feature sapphire pistons, stainless steel cylinders, and return valves equipped with sapphire balls and stainless steel seats. For analytical purposes, HPLC pumps should have flow rates ranging from 0-10 ml/min. However, for preparative HPLC, flow rates exceeding 100 ml/min may be necessary. Various pump types are available to provide the required pressures and flow rates for modern liquid chromatography. In the early years of the LC renaissance, two common pump types were the pneumatic pump, achieving high pressures through pneumatic amplification, and the syringe pump, essentially a large, robust syringe with a motor-driven plunger. Nowadays, the majority of modern HPLCs are equipped with reciprocating pumps featuring either pistons or diaphragms.

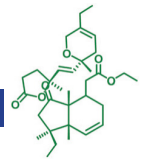
SINGLE PISTON RECIPROCATING PUMP

The single-piston reciprocating pump was the pioneering type employed with high-efficiency LC columns (columns packed with small particles) and remains widely used today. It boasts a straightforward design and is relatively cost-effective. The diagram of the single-piston pump is illustrated in the following figure.



Single Piston Reciprocating Pump

Most pistons in modern LC pumps are crafted from synthetic sapphire to minimize wear and prolong the pump's operational lifespan. The cylinder is typically constructed of stainless steel and is connected to two non-return valves aligned with the inlet and outlet connections to the pump. The piston is propelled by a stainless-steel cam, which pushes the piston into the cylinder, expelling the solvent through the exit non-return valve. After reaching its maximum



movement, the piston follows the cam and returns due to the pressure exerted by the return spring. During this motion, the cylinder is replenished with more solvent through the inlet non-return valve. The cam's shape is designed to ensure a linear movement of the piston during solvent expression, followed by a sudden return movement on the refill stroke. This design minimizes the pulse effect resulting from the refill action.

RAPID REFILL PUMP

To mitigate the refill pulses associated with a single-piston pump, various rapid refill systems have been devised. These designs have encompassed ingeniously crafted actuating cams to propel the piston swiftly during the refill mode to electronically controlled piston movements.

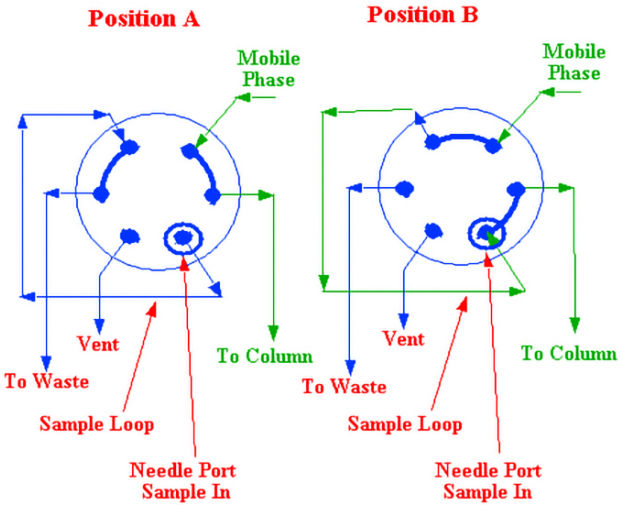
DIAPHRAGM PUMP

The distinctive feature of the reciprocating diaphragm pump lies in the fact that the actuating piston does not directly interact with the mobile phase. As a result, the demands on the piston-cylinder seal are less significant. The diaphragm boasts a relatively large surface area, leading to a relatively small movement, allowing the pump to operate at a relatively high frequency.

HPLC SAMPLE VALVES

As sample valves are positioned between the pump and the column in HPLC, it is imperative for HPLC sample valves to withstand pressures up to 10,000 PSI. In analytical HPLC, the sample volume should be adjustable from sub-microliters to a few microliters, whereas in preparative HPLC, the sample volume may exceed 10 ml. The higher the operating pressure, the more tightly the valve seating surfaces must be pressed together to eliminate any leaks. Any abrasive material, no matter how fine, that passes into the valve can potentially score the valve seating each time it is rotated, leading to leaks. This can result in varying sample sizes between injections and ultimately impact the accuracy of the analysis. In LC, the sample valve incorporates an additional loading port and functions as an internal loop valve. The key distinction from a regular external loop sample valve is the addition of an extra port at the front of the valve. This extra port allows direct injection of a sample using a syringe into the front of the sample loop. At position (A), the inject position, the sample flows into the sample loop. The needle tip passes through the rotor seal and, upon injection, directly contacts the ceramic stator face. After injection, the valve is rotated to position (B), and the mobile phase flushes the sample directly onto the column. The sample is forced out of the beginning of the loop, avoiding the need to flow through the entire length of the loop. This injection system is well-suited for quantitative LC and is likely the most widely used injection system

.



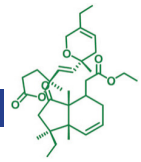
External Loop Sample Valve

HPLC COLUMNS

HPLC columns are filled with extremely fine particles, typically a few microns in diameter. These fine particles are necessary to achieve the low dispersion required for the high plate counts expected in modern HPLC. While modern columns can potentially reach plate counts exceeding 25,000 plates per column, such very high efficiencies are rarely observed with real samples. This is due to the dispersion associated with injection valves, detectors, data acquisition systems, and the dispersion caused by the higher molecular weight of real samples compared to common test samples. In general, LC columns achieve separation by exploiting the distinct intermolecular forces between the solute and the stationary phase, as well as those between the solute and the mobile phase. The column retains substances that interact more strongly with the stationary phase than those that interact more strongly with the mobile phase. Particularly, optically pure compounds can be utilized to create Chiral HPLC stationary phases.

LIQUID CHROMATOGRAPHY STATIONARY PHASES

Historically, the stationary phase employed in LC has been silica gel, which primarily separates solutes based on polarity. However, owing to its distinctive structure, silica gel also displays pronounced exclusion characteristics. Bonded phases were subsequently introduced to offer a material capable of separating solutes through dispersive interactions and to provide



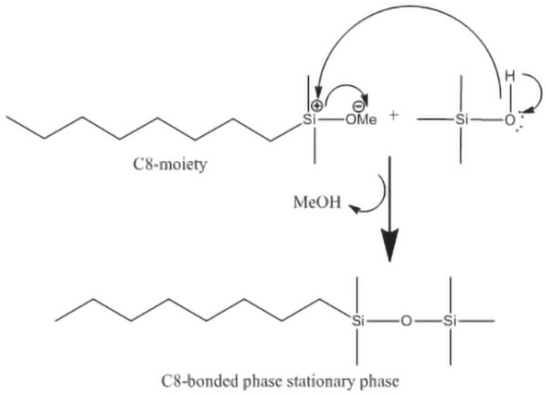
semi-polar stationary phases. These bonded phases were also silica gel-based. More recently, polymeric stationary phases were introduced to furnish materials that are insoluble in water and exhibit stability under extreme pH conditions.

THE STRUCTURE OF SILICA GEL

The primary silica gel particle matrix comprises a core of silicon atoms connected by siloxane bonds (silicon-oxygen-silicon bonds). On the surface of each primary particle, some residual uncondensed hydroxyl groups from the original polymeric silicic acid persist. There are three types of hydroxyl groups. The first is a single hydroxyl group attached to a silicon atom that has three siloxane bonds connecting it to the gel matrix. The second is one of two hydroxyl groups attached to the same silicon atom, which, in turn, is linked to the matrix by only two siloxane bonds. These twin hydroxyl groups are referred to as geminal hydroxyl groups. The third is one of three hydroxyl groups attached to a silicon atom that is now connected to the silica matrix by only a single siloxane bond.

BONDED PHASES

Bonded phases are created by reacting the surface hydroxyl groups with a suitable reagent to chemically link an organic moiety to the silica surface. The type of interaction between the solute and the surface is determined by the nature of the organic moiety. The most efficient bonded phase achieves maximum surface coverage. However, due to steric hindrance from the bonded moiety, only a proportion of the silanol groups can be bonded, and addressing this issue is challenging. Incomplete silanization can occur when the reagent molecule is excluded from the smaller pores of the silica. This exclusion is particularly problematic when bonding relatively large molecular weight materials, such as long-chain hydrocarbons, onto the silica surface. Therefore, selecting a silica gel with a relatively large pore size (e.g., a mean pore diameter of 150 Å) is crucial. This choice may limit the surface area to between 150 and 250 sq.m per gram, thereby reducing the retentive capacity of the stationary phase. Aromatic hydrocarbons, like toluene (boiling point: 110˚C), or mixed xylenes (boiling range: 138-140˚C), are typically used as solvents in bonded phase synthesis. The synthesis procedure may vary depending on the batch size and the type of silanizing reagent. The method for synthesizing bonded phases with alkoxysilane reagents is illustrated below. The most reactive alkoxy reagents are methoxy and ethoxysilanes, and their reaction with a hydroxyl group releases methanol or ethanol.



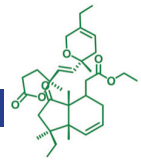
The concluding capping process is identical to that utilized in the method employing chlorosilanes reagents, employing hexamethyldisilazane as the capping reagent. Alkoxy-silanes are nearly as accessible as chlorosilanes and are more straightforward and agreeable to handle.

LC MOBILE PHASES

The selection of a phase system can be quite intricate, especially when dealing with multicomponent mixtures. Initially, the choice of the stationary phase needs consideration, and this decision must be guided by the interactive nature of the solutes intended for separation. If the solutes are mainly dispersive, the stationary phase must also be dispersive (reversed phase) to facilitate dispersive interaction with the solutes and ensure adequate retention and selectivity. In cases where the solutes are highly polar, a polarizable stationary phase (containing aromatic rings or cyano groups) would be suitable for separating the solutes through polar and induced polar interactions. For weakly polar solutes, a strong polar stationary phase, such as silica gel, would be necessary for separation based on polar interactions.

COLUMN OVENS

The impact of temperature on LC separations is often not as profound as its influence on GC separations but can be crucial when closely similar substances are being separated. In LC, a change in temperature alters the free energy of the solute in both phases (typically in a corresponding manner). Consequently, the net change in the free energy difference with temperature, which governs the absolute retention magnitude, may be relatively small. However, its effect on relative retention can be highly significant and, in fact, serve as the determining factor in achieving a satisfactory resolution. An increase in temperature enhances the diffusivity of the solute in both phases, thereby increasing dispersion due to longitudinal diffusion and decreasing dispersion caused by resistance to mass transfer.



HPLC DETECTORS

Over the past thirty years, numerous LC detectors have been developed based on various sensing principles. However, only about twelve of them prove effective for LC analyses, and among those twelve, only five are commonly utilized. The predominant detectors in LC analysis include the UV detector (both fixed and variable wavelength), the photo diode array detector, the electrical conductivity detector, the fluorescence detector, and the refractive index detector. These detectors find application in over 95% of all LC analytical scenarios.

THE UV DETECTOR

The UV detector stands out as the most popular and valuable LC detector currently accessible to analysts. While the UV detector has certain limitations, especially when detecting nonpolar solutes lacking UV chromophores, it boasts the optimal combination of sensitivity, linearity, versatility, and reliability among all developed LC detectors. Multi-Wavelength UV detectors employ a single wavelength to detect the solute. Additionally, most multi-wavelength UV detectors can furnish a UV spectrum of the eluted solute when appropriately configured.

ELECTRICAL CONDUCTIVITY DETECTORS

The electrical conductivity detector is limited to detecting substances that ionize; hence, it is often employed in the analysis of inorganic acids, bases, and salts. Additionally, it has proven particularly useful in detecting organic acids and bases, commonly needed in environmental studies and biotechnology applications. The sensor is the most straightforward among all detectors, comprising only two electrodes situated in a suitable flow cell.

THE FLUORESCENCE DETECTOR

The fluorescence detector is among the most sensitive LC detectors, making it suitable for trace analysis. However, its sensitivity comes with the limitation of a linear response over a relatively narrow concentration range. The detector can be assumed to have a linear response over a concentration range of only two orders of magnitude. Unfortunately, the majority of substances do not naturally fluoresce, posing a significant drawback to this type of detector. Consequently, in many cases, fluorescent derivatives must be synthesized to make the substances of interest detectable.

THE REFRACTIVE INDEX DETECTOR

The refractive index detector is among the less sensitive LC detectors. It is highly responsive to variations in ambient temperature, pressure, and flow rate, making it unsuitable for gradient elution. Despite these numerous drawbacks, this detector proves extremely valuable for

detecting nonionic compounds that neither adsorb UV light nor fluoresce.

HPLC DATA ACQUISITION

The detector output, typically in millivolts, undergoes conversion through a scaling amplifier to generate a voltage compatible with the analog-to-digital (A/D) converter. The A/D converter transforms the voltage output into a binary number, which is temporarily stored in a register. This process iterates continuously at a specified rate known as the 'sampling rate.' The computer regularly samples the current binary number stored in the register and stores this information, usually on a hard disk. Upon completion of the analysis, the computer retrieves all the stored data, computes the retention report, compares peak heights or areas, and provides quantitative analysis based on the processing program in use. The results are then printed out in tabulated form. Modern data processing software often integrates routines capable of handling chromatograms with incompletely resolved sample components. These routines deconvolute individual peaks from composite envelopes, calculating the area of each deconvoluted peak. While these algorithms are effective for peaks within the tail of a major peak, they may be less accurate for composite envelopes with numerous unresolved peaks.

SUGGESTED READINGS

Baianu, I. C., Costescu, D., Hofmann, N. E., Korban, S. S. Infrared Chemical Imaging and Fluorescence Microspectroscopy. 2004., q-bio/0407006 (July 2004).

Beesley T. E., & Scott R. P. W. (1998). Chiral Chromatography, John Wiley and Sons, Chichester-New York, 39.

Dandenau, R. D., & Zenner, E. M. (1979). Journal of High Resolution Chromatography, 2, 351.

Desty, D. H., Goldup A., & Wyman, B. F. (1959). Journal of the Institute of Petroleum, 45, 287.

Drago, R. S. (1992). Physical Methods for Chemists, Surfside Publishing, 1992.

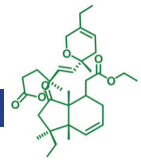
Dubois, J., Sando, G., Lewis, E. N., G.I.T. Laboratory Journal Europe, No. 1-2, 2007.

Harley, J ., Nel, W., & Pretorious, V. (1958). Nature, London, 181, 77.

Harris, D. C., & Bertolucci, M. D. (1978). Symmetry and Spectroscopy, Dover, 1978.

James A. T., & Martin, A. J. P. (1952). Biochemical Journal, 50 (1952) 679.

James, A. T. (1966). The Times Science Review, Summer, 8.



Katz E., & Scott R. P. W. Journal of Chromatography, 253, 159.

Lide, D. R. CRC Handbook of Chemistry and Physics, 75th ed. (Boca Raton, FL: CRC Press, 1994), 9–79.

Luthria, D. Oil Extraction and Analysis. pp.241-273, AOCS Press., Champaign, IL, 2004.

Martin A. J. P., & Synge R. L. M. (1941). Biochemical Journal, 35, 1358.

Nakanishi, K., Berova, N., & Woody, R. W., Circular Dichroism, VCH Publishers, 1994.

Ogan, K. L., Reese, C., Scott, R. P. W. (1982). Journal of Chromatographic Science, 20, 425.

Pasto, D. J., & Johnson, C. R. (1969). Organic Structure Determination, Prentice-Hall, 1969.

Schmidt G.J., & Scott R.P.W. (1985). Analyst, 110, 757.

Scott R. P. W. (1999). Analyst, 124.

Scott R. P. W., & Beesley T. E. (1999) Analyst, 124, 713

Scott R.P.W. Chromatography Detectors: Design. Function and Operation, Marcel Dekker Inc., New York-Basle (1997).

Tiselius A., Claesson D. (1942). Ark. Kemi. Mineral. Geol. 15B(1942).

Tswett M.S. (1905). Tr. Protok . Varshav . Obshch . Estestvoispyt Otd . Biol . 14.

Williams, D. H., & Fleming, I. (1987). Spectroscopic methods in organic chemistry, McGraw-Hill, 1987.

Willstatter R., Stoll A. (1913). Utersuchungenuber Chlorophy , Springer, Berlin.